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Role of neutrophil derived oxidants and elastase in lipopolysaccharide-mediated renal injury

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Role of neutrophil derived oxidants and elastase in lipopolysaccharide-mediated renal injury. Gram-negative bacterial sepsis is frequently associated with acute renal failure but the specific effects of lipopolysaccharide (LPS) and other bacterial products on kidney function are not known. Since either LPS or formyl-methionyl-leucyl-phenylalanine (FMLP)—a chemotactic peptide from bacterial cell walls—activate neutrophils (PMN) to release a number of potentially toxic factors in vitro, we determined the effect of adding PMN with LPS and/or FMLP to isolated perfused rat kidneys. Isolated rat kidneys perfused with LPS alone or LPS and normal PMN had normal glomerular filtration rates (GFR) and tubular Na reabsorption (T_{Na}). Kidneys perfused with FMLP alone or FMLP and normal PMN also had normal GFR and T_{Na}. In contrast, addition of PMN with both FMLP and LPS caused progressive renal dysfunction. For example, after 60 minutes of perfusion, GFR was reduced from 610 ± 31 to 147 ± 17 μl/min/g and T_{Na} from 97 ± 1 to 72 ± 2%, both $P < 0.01$. Perfusion with the O_2 metabolite scavengers catalase or dimethylthiourea afforded no protection while perfusion with the neutrophil elastase inhibitor Eglin C conferred substantial, but not complete, protection: GFR 492 ± 34 μl/min/g; T_{Na} 91 ± 3%. However, perfusion with both Eglin C and catalase completely prevented the toxic effects of LPS and FMLP-treated PMN on renal function. We conclude that in isolated kidneys, 1) the toxic effects of LPS requires FMLP-treated PMN and that 2) LPS and FMLP treated PMN cause progressive renal injury which is mediated by both O_2 metabolites and neutrophil elastase.

Gram-negative bacterial sepsis is frequently associated with acute renal failure [1]. Bacterial endotoxin appears to be pivotal in the pathogenesis of renal failure since infusion of the lipopolysaccharide (LPS) fraction of endotoxin (which possesses most of the biologic activity) [2] results in marked reductions in GFR [3]. The mechanism by which LPS results in renal dysfunction appears to be complex. Although bacterial sepsis is often associated with systemic hypotension, acute renal failure can occur in the absence of changes in systemic hemodynamics [3–5]. A number of additional factors have been implicated to account for reductions in GFR. These include renal nerves, hormones such as angiotensin II and norepinephrine [3, 6], eicosanoids [3, 6, 7], cytokines such as tumor necrosis factor [8] or interleukin-1 [9] and platelet activating factor [10].

LPS and other bacterial products are potent stimulators of polymorphonuclear leukocytes (PMN) in vitro [11–14]. Activated PMN release a number of factors which are potentially nephrotoxic. These include toxic products of oxygen metabolism (O_2 metabolites) as well as proteinases such as neutrophil elastase and metalloproteinases [15, 16]. Despite this potential interaction between LPS and PMN, the role of PMN in acute renal failure associated with gram negative sepsis has not been carefully evaluated. Moreover, while synergism between neutrophil elastase and oxidants has been described in other systems [16, 17], this type of additive interaction between neutrophil derived oxidants and proteinases has not been described in the kidney. We undertook this study, therefore, to determine the role of PMN in bacterial-product mediated renal injury. To address this issue, we utilized the isolated perfused rat kidney. In this simple system, the specific effects of PMN activated with bacterial products-LPS and/or formyl-methionyl-leucyl-phenylalanine (FMLP) can be determined in the absence of circulating hormonal or other factor which would otherwise complicate elucidation of the role of PMN. Using this strategy, we obtained results which support the premise that bacterial products activate PMN to injure kidneys by both O_2 metabolite- and elastase-mediated mechanisms.

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Methods

Sources of reagents

The following reagents were used in this study: albumin (fraction V bovine, Reheis Chemical, Phoenix, Arizona, USA); hydroxyl [^{14}C] methyl inulin (Amersham, Arlington Heights, Illinois, USA); dimethylthiourea (DMTU) (Alfa Products, Danvers, Massachusetts, USA); phorbol myristate acetate (PMA), catalase (CAT) (bovine liver, 2,000 U/mg protein), FMLP, elastase (Type IV Pancreatic 70 U/mg protein (Sigma Chemical, St. Louis, Missouri, USA); LPS (#201 *Escherichia Coli* 0111: B4, List Biological Laboratory, Inc., Campbell, California, USA); Eglin C (Ciba-Geigy, Switzerland) [17, 18].

Perfusion of isolated rat kidneys

Perfusion of the isolated rat kidney was conducted according to Nishiitsutsuji-Uwo, Ross and Krebs [19] as modified by Little and Cohen [20] and previously described in our laboratory [21]. Briefly, after the right renal artery was cannulated, the kidney was transferred to a perfusion chamber and perfused using a pulsatile pump at a constant mean arterial pressure (distal to the tip of the cannula) of 100 mm Hg. Following a 15-minute equilibration period, three urine collections and perfusion samples were obtained at 15 minute intervals. Perfu-

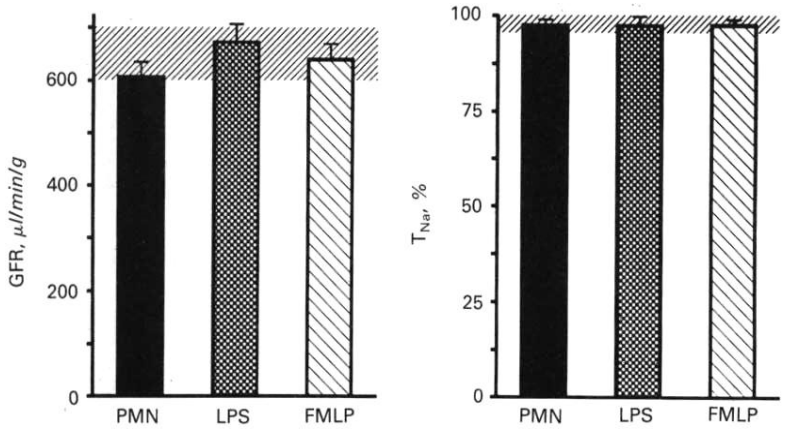

Fig. 1. *Effect of addition of neutrophils (PMN N = 5), chemotactic peptide (FMLP, N = 5) or lipopolysaccharide (LPS, N = 6) on the function of isolated perfused kidneys.* Data are presented after 60 minutes of perfusion. Grey area denotes mean value ± SEM (N = 10) for kidneys perfused without additions (control kidneys).

sion samples were placed in a test tube for subsequent determinations of sodium and [^{14}C] inulin. Each perfusion lasted for 60 minutes. Urine and perfusate samples were stored at −20°C within 60 minutes of collection. For each perfusion, 150 ml of a Krebs-Ringer bicarbonate solution was used that contained urea, (hydroxyl [^{14}C] methyl) inulin, and albumin. The final composition of the perfusion medium (in mM unless otherwise noted) was 140 Na, 5.0 K, 1.2 ionized Ca, 1 Mg, 105 Cl, 25 bicarbonate, 1 sulfate, 1 phosphate, 7 urea, 70 mg/ml albumin, 5 glucose, and 1 ml/100 ml perfusate of Aminosyn 8.5% (Abbott Laboratories, North Chicago, Illinois, USA).

Measurement of renal injury

Measurements of glomerular filtration rate (GFR), and tubular sodium reabsorption (T_{Na}), perfusion flow rate (PFR) were used to assess renal injury. Urinary clearances of [^{14}C] inulin and sodium were calculated from their respective urine and plasma concentration ratios. Sodium was measured with an IL 343 flame photometer (Instrumentation Laboratory, Lexington, Massachusetts, USA). Radioactivity of [^{14}C] inulin was counted in a Packard Tricarb model 460 liquid scintillation counter (Packard Instruments, Laguna Hills, California, USA).

Preparation of neutrophils

Neutrophils were purified from human blood anticoagulated with sodium citrate (0.38%). Cells were separated by sedimentation and centrifugation and then were resuspended in platelet-poor plasma, diluted 1:4 with 6% dextran (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA) in saline. Erythrocytes were separated from leukocytes by sedimentation. The leukocyte-rich supernatant was aspirated, underlaid with Ficoll-Hypaque (Pharmacia Fine Chemicals), and centrifuged at 275 g to separate monocytes and neutrophils. Sedimented neutrophils were resuspended in Hank's buffered salt solution (HBSS) before injection into perfusates. Final preparations contained 94 to 96% neutrophils and 4 to 6% monocytes.

Preparation of phorbol myristate acetate (PMA)

PMA (12-O-tetradecanoyl-phorbol-13-acetate) was dissolved in dimethylsulfoxide at 5 mg/ml and divided into 200 μg aliquots that were frozen at −70°C. Just before use, aliquots were thawed and diluted to 200 μg/ml in normal saline.

Our investigations consisted of four protocols:

Protocol I was designed to determine the effects of LPS, PMN and FMLP on isolated kidney function. Isolated kidneys were perfused with LPS (1 μg/ml, N = 6), PMN (3×10^5 cells/ml, N = 5) or FMLP (1×10^{-6} M; N = 5). These concentrations were chosen because in preliminary studies, we determined that this combination of agents resulted in a very reproducible model of renal injury. PMN, LPS and FMLP were added to the perfusate at time 0 of perfusion.

Protocol II was designed to determined whether addition of PMN along with LPS and/or FMLP caused renal injury. For these studies, kidneys were perfused with PMN and LPS (N = 5), PMN and FMLP (N = 5), or PMN plus LPS and FMLP (N = 7). In other studies, kidneys were perfused with LPS and FMLP without PMN (N = 3).

Protocol III was designed to determine the role of O_2 metabolite scavengers in kidneys exposed to PMN plus LPS and FMLP. Isolated kidneys were exposed to PMN plus LPS and FMLP in the presence of no additions (N = 5) or DMTU (15 mM, N = 6) or CAT (200 U/ml, N = 6).

Protocol IV was designed to determine the contribution of neutrophil elastase to injury in kidneys exposed to PMN plus LPS and FMLP. For these studies, the non-oxidizable neutrophil elastase inhibitor Eglin C [17, 18] was utilized. To determine the specificity of Eglin C, kidneys were perfused with elastase (2.8 U/ml, N = 3 and 5.6 U/ml, N = 4), or elastase and Eglin C (2 μM) together (N = 6) or elastase plus CAT (N = 4) or elastase plus DMTU (N = 4). In additional studies, kidneys were exposed to PMN with PMA (10 ng/ml) in the presence of no additions (N = 5), Eglin C (N = 5) or CAT (N = 6). In the final group of perfusions, isolated kidneys were exposed to PMN plus LPS and FMLP with no additions (N = 6) or Eglin C (N = 6) or Eglin C plus CAT (N = 6).

Statistical analyses

Statistical analyses were performed using one-way analysis of variance in conjunction with Scheffe's-test for comparison of multiple means. A P value of < 0.05 was considered significant. Data were expressed as means ± SEM.

Results

Figure 1 shows the effect of addition of PMN, LPS or FMLP to isolated perfused kidneys. During the 60 minutes of perfu-

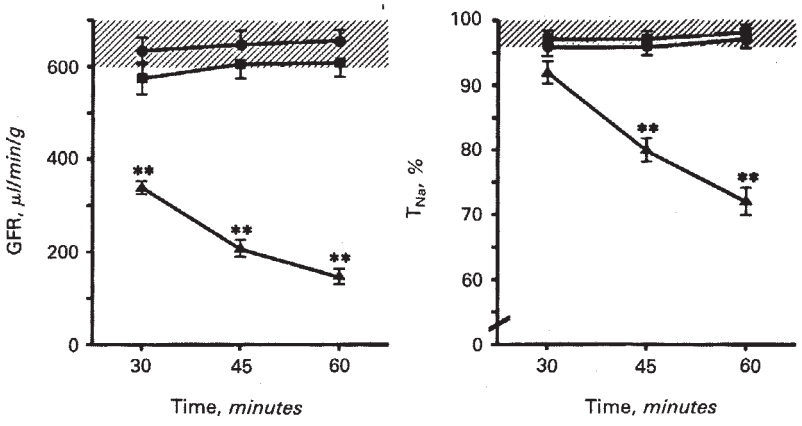

Fig. 2. *Effect of addition of PMN plus LPS (●, N = 5), PMN plus FMLP (■, N = 5) or PMN plus LPS and FMLP (▲, N = 7) on the function of isolated perfused kidneys.* Grey area denotes mean value ± SEM ($N = 10$) for control kidneys $^{**}P < 0.01$ vs. kidneys perfused with PMN and either LPS or FMLP.

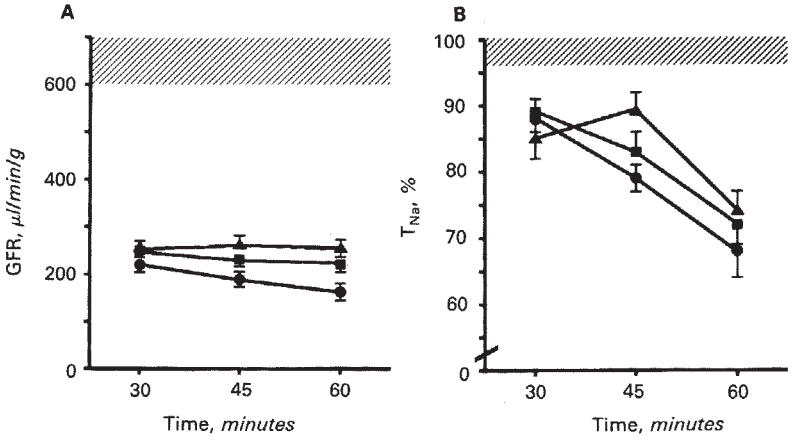

Fig. 3. *Effect of no additions (●, N = 5), addition of catalase (■, CAT, N = 6) or dimethylthiourea (▲, DMTU, N = 6) on kidneys perfused with PMN, LPS and FMLP.* Grey area denotes mean value ± SEM ($N = 10$) for control kidneys.

sion, there were no effects attributable to addition of PMN, LPS or FMLP alone on GFR, T_{Na} or PFR (data not shown).

Figure 2 shows the effects of addition of PMN with LPS and/or FMLP on renal function. Kidneys exposed to PMN and either LPS or FMLP did not have decreases in GFR, T_{Na} or PFR (data not shown). In contrast, there were time-dependent, progressive decreases in GFR and T_{Na} (but not PFR) in kidneys exposed to PMN along with both LPS and FMLP. For example, after 60 minutes of perfusion, GFR was reduced to 147 ± 17 μl/min/g and T_{Na} 72 ± 2% (both $P < 0.01$) in kidneys exposed to PMN plus LPS and FMLP compared to kidneys exposed to PMN and FMLP (610 ± 31 μl/min/g and 98 ± 1%) or PMN and LPS (655 ± 25 μl/min/g and 97 ± 1%). Addition of LPS and FMLP without PMN did not result in decreases in GFR (593 ± 26 μl/min/g) or T_{Na} (97 ± 2%).

To determine if decreases in renal function in kidneys exposed in PMN in the presence of LPS and FMLP were caused by O_2 metabolites, O_2 metabolite scavengers, catalase or DMTU, were included in the perfusate. In earlier studies, we found that the concentrations of CAT and DMTU used in these studies prevented renal dysfunction caused by PMN stimulated with PMA, a known agonist of O_2 metabolite release by PMN [22]. Figure 3 shows that either CAT or DMTU resulted in small, but statistically insignificant, increases in both GFR and T_{Na} compared to kidneys perfused without additions. Addition of CAT or DMTU alone during perfusion did not alter kidney function.

To determine whether neutrophil elastase mediated decreases in renal function in kidneys perfused with PMN treated with LPS and FMLP, studies were performed with the potent non-oxidizable neutrophil elastase inhibitor, Eglin C [17, 18]. Figure 4 shows the effect of elastase on isolated kidney function. Perfusion with elastase caused time and concentration dependent decreases in GFR and T_{Na} (but not PFR, data not shown). For example, during perfusion with elastase (5.6 U/ml), GFR was 443 ± 23, 318 ± 24 and 221 ± 22 μl/min/g after 30, 45 and 60 minutes of perfusion, respectively. Elastase mediated renal injury was prevented by co-addition of Eglin C but not by the O_2 metabolite scavengers CAT or DMTU. Addition of Eglin C alone during perfusion did not alter the function of isolated kidneys.

Figure 5 addresses the specificity of Eglin C treatment in kidneys perfused with PMN and PMA. Under these conditions, renal injury was prevented by addition of catalase but not Eglin C. These results indicate that Eglin C prevents elastase, but not O_2 metabolite effects on isolated kidneys.

Figure 6 demonstrates the effect of Eglin C in kidneys exposed to PMN added with LPS and FMLP. Compared to no additions, perfusion with Eglin C caused increases in GFR and T_{Na} which exceeded values in kidneys perfused with PMN,

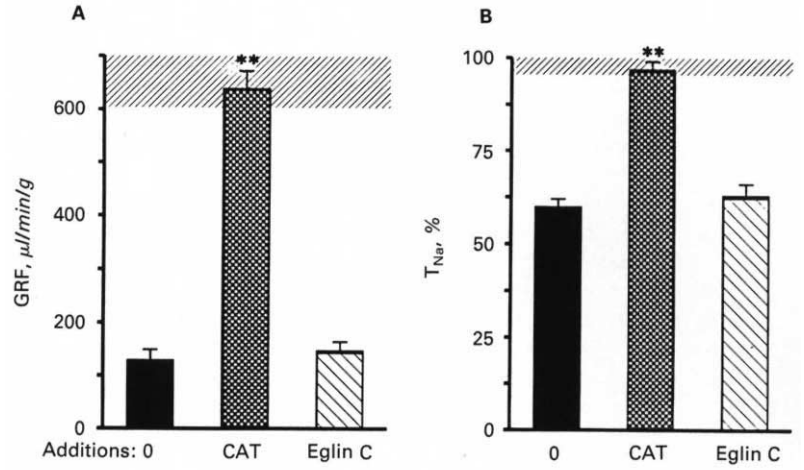

Fig. 4. *Effect of no additions or addition of Eglin C, (N = 4) or catalase (CAT, N = 4) or dimethylthiourea (DMTU, N = 4) on renal injury caused by low (2.8 U/ml) and high (5.6 U/ml) concentrations of elastase.* Results are presented after 60 minutes of perfusion, $^{*}P < 0.01$ compared to elastase alone. Grey area denotes mean value ± SEM ($N = 10$) for control kidneys.

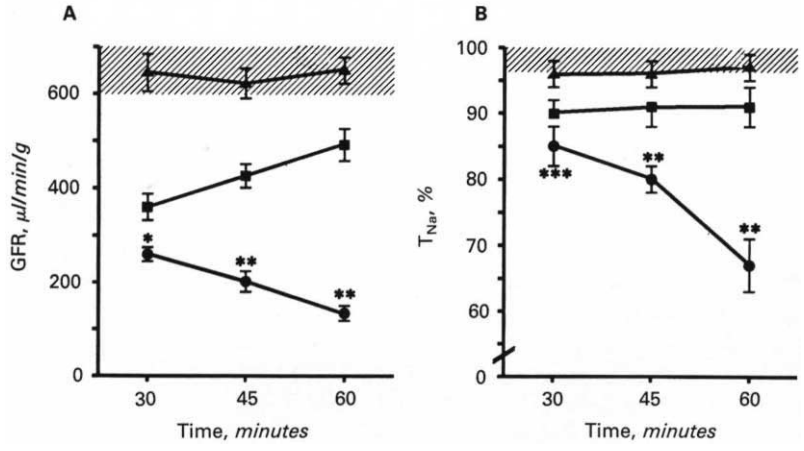

Fig. 5. *Effect of no additions (N = 5), addition of catalase (CAT, N = 6), or Eglin C (N = 5) on kidneys perfused with phorbol myristate acetate (PMA) and PMN.* Results are presented after 60 minutes of perfusion. Grey area denotes mean value ± SEM ($N = 10$) for control kidneys. $^{**}P < 0.01$ compared to PMA and PMN.

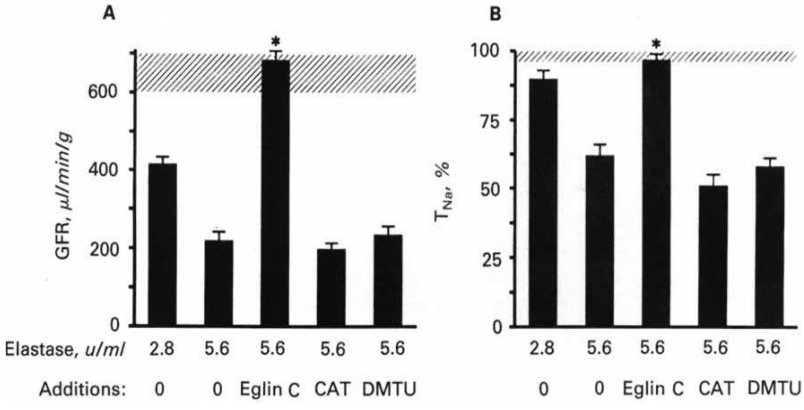

Fig. 6. *Effect of no additions (●, N = 6), addition of Eglin C (■, N = 6) or Eglin C and CAT (▲, N = 6) on kidney perfused with PMN, LPS and FMLP.* Grey area denotes mean value ± SEM ($N = 10$) for control kidneys. $^{*}P < 0.05$ vs. Eglin C, $P < 0.01$ vs. Eglin C plus CAT. $^{**}P < 0.01$ vs. Eglin C and Eglin C plus CAT. $^{***}P$ = NS vs. Eglin C, $P < 0.05$ vs. Eglin C plus CAT

LPS and FMLP but did not reach control values. To determine whether O_2 metabolites contributed to residual decreases in renal function in kidneys perfused with Eglin C, CAT was included with Eglin C. The figure demonstrates that compared to no additions, perfusion with Eglin C and CAT normalized GFR and T_{Na} measurements in kidneys perfused with PMN, LPS and FMLP.

Discussion

Systemic administration of LPS causes renal dysfunction in vivo by unknown mechanisms which could include alterations in systemic or renal hemodynamics [3–5], activation of renal nerves [3] or alterations in circulating hormones and cytokines [3, 5–10]. We used the isolated perfused kidney so that we could

determine the specific effects of LPS in a relatively simple test system. As has also recently been reported by Cohen, Black and Wertheim [23], we found that addition of LPS had no adverse effect on renal function. Only when LPS was added with FMLP-treated PMN did injury develop in the isolated perfused kidney.

In earlier studies [22] and other [24, 25] have shown that PMN stimulated with a single agent such as PMA cause renal injury. In the current studies we were not able to demonstrate deleterious effects of LPS or FMLP and PMN. However, concentrations of LPS, which by themselves or with PMN did not injure kidneys, appeared to enhance the injurious effect of FMLP-treated PMN. A similar phenomenon is observed in vitro. Under these conditions, LPS appears to enhance the release of a number of PMN products. For example, concentrations of LPS which do not cause oxidant or leukotriene B_4 release, enhance the effect of formyl peptides to cause superoxide release [11] and zymogen to cause leukotriene B_4 release from PMN [26]. Although the precise mechanism of PMN priming is not known, recent studies suggest that increases in cytosolic calcium are an important mediator of LPS priming of PMN for an enhanced superoxide release [27].

The model used in the current studies may be relevant to clinical sepsis. In this regard, in gram negative sepsis circulating PMN are exposed to low concentrations of multiple PMN activators including LPS and chemotactic peptides. Whereas neither agonist circulates in concentrations sufficient to activate PMN, Henson et al have shown that incubation of PMN with LPS results in enhanced release of both oxidants and neutrophil elastase in response to concentrations of a second agonist which when administered alone, do not activate PMN [11, 13, 28]. Our results in the isolated perfused kidney are consistent with the data of Henson et al [11, 13, 28] and indicate that LPS-mediated renal injury requires FMLP-activated PMN.

Although our data reveal an important role for PMN in LPS-mediated renal injury, other circulating leukocytes may also contribute to LPS injury. For example LPS stimulates monocytes to release a variety of biologically active cytokines such as tumor necrosis factor [29, 30], which have been proposed to mediate many of the responses associated with endotoxemia [31].

In addition our data imply that LPS injury is mediated by the effects of LPS on PMN rather than on kidney tissue. However, the possibility that LPS interacted with kidney tissue to activated PMN cannot be completely excluded since other PMN activators directly injure epithelial cells and endothelial cells [32–34]. In this scheme, LPS would injure epithelial and/or mesangial cells making them susceptible to PMN. Although we cannot exclude a subtle effect of LPS or FMLP on the kidney, our results underscore the critical importance of PMN in this model of renal failure since addition of LPS or FMLP in combination with PMN did not result in renal injury. Renal injury only occurred when PMN were exposed to both LPS and FMLP.

Activated PMN release a number of products which could potentially injure kidneys. Neutrophil derived toxins include O_2 metabolites, myeloperoxidase and proteinases, such as neutrophil elastase and metalloproteinases [15, 16]. O_2 metabolites from PMN can injure kidneys directly [35] and activate latent metalloproteinases which can injure kidneys [24]. Myeloperoxidase can interact with circulating halides and hydrogen peroxide to cause marked increases in glomerular protein excretion [36]. Neutrophil elastase appears to be an important mediator of lung injury especially in settings where alpha-1-antiprotease inhibitors have been inactivated. For example, perfusion of isolated lungs with purified neutrophil elastase increased lung permeability but only after addition of H_2O_2 to reduce anti-elastolytic activity [37]. Our data suggest that neutrophil elastase can now be added to the list of PMN products which are capable of causing glomerular and tubular dysfunction since perfusion with the specific neutrophil elastase inhibitor Eglin C partially prevented LPS/FMLP/PMN-induced renal injury.

Depending on the selectively of the stimulus used to activate PMN, the role of oxidants, myeloperoxidase and proteinases in renal injury may vary. For example, PMA is a relatively good stimulator of O_2 metabolite release compared to its effect on proteinase release [13]. By comparison, FMLP is a relatively potent stimulus for degranulation compared to its ability to initiate O_2 metabolite generation by PMN [13, 28]. LPS/FMLP, in the concentrations use in our study, appears to release both O_2 metabolites and neutrophil elastase. Thus injury stimulated by PMA may be more susceptible to O_2 metabolite scavengers (Fig. 5) while injury caused by LPS/FMLP (Fig. 6) appears to require both O_2 metabolite and neutrophil elastase inhibitors for reversal.

Although both O_2 metabolites and neutrophil elastase appeared to contribute to LPS/FMLP and PMN mediated renal injury, the toxic effects of LPS/FMLP/PMN on renal function could not be totally reversed by addition of either the neutrophil elastase inhibitor or oxygen metabolite scavengers alone. Instead, perfusion with both O_2 metabolite scavengers and the neutrophil elastase inhibition was required to prevent LPS/PMN renal injury. O_2 metabolites and neutrophil elastase could interact to cause renal injury in several ways. Shah has shown that O_2 metabolites activate latent metalloproteinases to cause glomerular basement membrane degradation [24]. This would not seem to be an important effect of O_2 metabolites on neutrophil elastase since this enzyme is released in an active form and does not require further modification for function. Alternatively, O_2 metabolites might enhance the action of neutrophil elastase by inactivating circulating antiproteinases [16, 37]. The major anti-elastolytic activities in serum are α_1 proteinase inhibitor and α_2 macroglobulin. Both of these compounds are susceptible to oxidant induced inhibition of action [16]. Thus O_2 metabolites produced by PMN inactivate protective antielastolytic activities so that the toxic effect of neutrophil elastase is enhanced [16, 37].

In earlier studies we showed that O_2 metabolites generated by xanthine oxidase caused injury in reperfused ischemic kidneys [38]. Additionally, we showed that normal PMN worsened reperfusion injury by an O_2 metabolite mediated mechanism [23]. The current studies expand on the possible role of O_2 metabolites and suggest a possible interaction between O_2 metabolites produced by ischemic kidneys and PMN activated with LPS. O_2 metabolites produced by kidneys during reperfusion could enhance the deleterious effects of neutrophil elastase in much the same way in which O_2 metabolites produced by LPS/FMLP-activated PMN enhance neutrophil elastase-mediated injury. This interaction may be particularly relevant to the clinical observation that renal ischemia predisposes to further

renal injury in many situations, including gram-negative bacterial sepsis.

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